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METHODS OF BACTERIOLOGICAL EXAMINATION OF MILK.

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THE bacteriological examination of milk is rapidly becoming one of the essentials in the maintenance of public health. For years milk supplies have been inspected and tested to chemical standards alone, with the result that watered, adulterated, and preserved milks have been practically driven from the markets. These chemical tests are necessary, and only fail in that they do not go far enough, since a milk impure and unwholesome on account of bacterial growth, or a milk from a diseased animal, cannot be detected by them.

The bacterial count and the microscopical examination of the milk sediment reveal whether the milk has been properly handled or not, and, to a great extent, the condition of the animal from which it came.

It so happened that a conviction of the importance of this examination came to several cities at about the same time, and as no methods of milk examination had been agreed upon among bacteriologists, each laboratory started the work independently as best it might. As a result, a large number of laboratories make routine bacteriological examinations of milk, but cannot compare results, because of differences in methods.

The subject is of so much importance that these various methods of technique and differences of apparatus ought to be compared, in order that the best may be chosen; and definite, uniform methods for routine examination should be agreed upon.

It is with this object in view that the following technique and apparatus, developed at the Boston Board of Health Laboratory, under the direction of Dr. H. W. Hill, are offered for comparison and criticism.

Before milk work was begun, Dr. E. H. Wilson, of Brooklyn, courteously permitted Dr. Hill to examine and collate the replies from about 15 prominent laboratories doing milk work, made

in response to a circular letter containing questions concerning technique, which Dr. Wilson had sent out. Although the replies showed wide differences in technique, even among workers devoted largely or solely to milk examinations, the technique in Boston was based on the consensus of the opinions given and modified from time to time as the work developed and became familiar to us.

For collecting samples, we have designed an apparatus using test tubes as containers; the samples, after thorough mixing, are transferred to the test tubes by means of large glass pipettes, a clean sterile pipette being used for each sample. The case for carrying the samples is made of copper, with double walls interlaid with half-inch felting, divided into three compartments, the central one for samples, the other two for ice. The test tubes are arranged in small racks, made of copper tubing weighted with a strip of lead and padded with rubber. Each rack holds four tubes. Holes in the bottoms of the partitions allow ice-water to circulate freely around the lower ends of the tubes. When this outfit is iced and closed, a constant temperature of 34° F. is maintained. The pipettes are carried beneath the sample case in a detachable copper box, adapted for sterilizing and divided into two compartments, the upper for clean sterile pipettes, the lower for the pipettes after use.

The use of the test tube has been adopted, in preference to the use of bottles, for the following reasons, dependent in most cases on the long slender shape of the tube:

1. Economy of floor area in the collecting case.
2. Avoidance of the necessity for two or more layers of containers, the lower layer of which would be always difficult of access.
3. The facility for maintaining low temperature by the circulation of ice-water about the lower ends of the tubes.
4. The ease with which all the usual washing, sterilizing, and general handling of test tubes can be done, since the test tube is a regular piece of ordinary apparatus, involving no departure from the ordinary routine in all the usual manipulations.

In plating, we have followed the methods recommended by the Bacteriological Committee.¹ We have found a dilution of 1:10,000 best suited for routine work. When examining samples recently

¹ *Jour. Infect. Dis.*, Suppl. No. 1, 1905, p. 1.

taken from individual cows or from a milk supply known to be fairly pure, we dilute 100 times. When the microscopical examination, which will be described in detail later, shows a milk crowded with bacteria, we dilute 1:1,000,000. The lowest count we have recorded is 300 bacteria to 1 c.c.; the highest 640,000,000.

For dilution water, square eight-ounce bottles have been found more easy to handle and more economical of space than other forms of bottles or flasks. Straight sided 1 c.c. pipettes are more easily handled than those with bulbs. They can easily be made from small glass tubing and calibrated in the laboratory.

For a medium, we use agar-agar made according to the directions of the Bacteriological Committee. A few changes have been made after careful comparison of results with varying acidities and percentages of agar. We have the best results with a 1 per cent agar, reaction + 1.5.

The additions of lactose or litmus to the medium has not, so far as we have tried it, proved of any special advantage.

We do not use gelatin, on account of the difficulty of maintaining uniform room temperature, and the length of time which must elapse before a report can be made.

The agar plates are incubated in a saturated atmosphere for 24 hours at 37° C.

Comparison of plates grown at room temperature with those grown at 37° C. has at times shown great differences in the number of colonies developing in duplicate plates. These differences, however, have not been uniform, the higher or lower temperature developing more colonies according to the nature of the organisms present. Incubation at 37° C. has been adopted, since it allows a much quicker report; it gives each sample the same treatment at a regulated temperature; and it allows a fair comparison of results obtained over long periods. If the plates are incubated 48 hours, a slightly higher average count will be obtained, not enough, however, materially to change the report. In many plates the count is lower at the end of 48 hours on account of small colonies becoming obscured in the growth of larger ones. There are also more spreaders, which means a greater loss of counts.

Porous Petri dish covers, suggested by Dr. Hill and since recom-

mended by the Bacteriological Committee, have proved very efficient in reducing the number of spreaders. By their use in our work, spreaders have been reduced from 35 per cent to practically none.

Spreading under glass covers seems to be caused principally by water condensing on the inner surface of the cover and on the surface of the agar. The first fault can be partially overcome by inverting the dish, but the second seems to be unavoidable except by using the porous covers. With the glass covers, the bacteria in the surface colonies multiply rapidly in the film of condensation water, often spreading over the whole surface, and thus making a count impossible. The dry porous earthenware covers absorb this condensation water, still leaving the atmosphere saturated, as is proved by the quick growth and large size of the colonies. It is not necessary to invert the dishes; the covers are cheaper and more durable than glass, and they can be marked with lead-pencil, the marks erasing easily. Organisms having an inherent tendency to spread from unusual motility are not prevented from spreading by the use of these covers.

With the porous covers, as with the glass, each additional day's incubation shows an increase in the number of spreaders, due partly at least to the fact that the moisture gradually fills the interstices so that they do not absorb as readily as at first; but, day by day, the spreaders are much smaller in number than with glass covers.

It is quite essential to the best results that the porous covers should be washed as seldom as possible. In sterilizing them, the process should be prolonged over the time necessary to kill the organisms, in order that the covers may be thoroughly dry.

Our counting apparatus is simple and inexpensive. A circle, four and one-half inches in diameter, divided into 10 equal segments, is cut into the surface of a child's school slate; the lines are then filled with red lead, against which any colonies lying immediately over them, are easily seen. The surface of the slate, which tends to become gray, with time and use, may be kept black by occasionally rubbing with a little vaseline. The Petri dish is placed, uncovered, bottom down over the circle. A wooden box, six by six by five inches, with open bottom, glass front, and a four-inch circular opening in the top, the wooden parts painted black within and without to avoid refraction of the light, is placed over the plate and centered.

A common four-inch reading glass, magnifying about two diameters, fits over the opening in the top of the box, thus protecting the plate, keeping a constant focus and leaving both hands of the operator free. A tally record for adding and recording the counts by a simple pressure of the thumb for each colony seen, completes the outfit.

The advantages of this counting apparatus may be summarized as follows:

1. Cheapness.
2. The lens is held at the proper focus, leaving both hands of the operator free.
3. The whole field is exposed to view, so that there is no danger of counting the same colony twice.
4. The radial division into 10ths makes it easy in a crowded plate to obtain an approximate estimate by multiplication of the count of one or two representative sections.
5. The plate is so well protected from contamination that it may be counted face up with the cover removed.

For the microscopic examination of milk we first obtain the sediment from a known quantity by centrifugalizing; Stewart, of Philadelphia, uses an apparatus by means of which a large number of samples may be treated at the same time.

A modification of this consists of an aluminum disc and cover, the whole being 10 inches in diameter and $\frac{5}{8}$ of an inch in depth.

This disc is fitted to hold 20 small tubes arranged radially. The tubes hold about 2 c.c. each. Both ends are closed with rubber stoppers. By the use of these tubes, the whole sediment from a known quantity of milk is obtained, and may be spread over a given space. We have arbitrarily adopted a space of 4 sq. cm.

For smearing the sediment, slides nine by two inches of common window glass are convenient. One of these may be ruled with blue pencil into 11 spaces, each 2 by 2 cm., leaving a space 2 by $1\frac{1}{2}$ cm. at the top of each for the sample number and a similar space at the lower end for comments on the microscopical examination. By stopping the tubes at one end before opening the sample case, the time of exposure of the samples to room temperature is lessened. Each sample need be exposed only a few seconds while filling the tube, and immediately replaced in the case.

The milk sample is shaken 25 times, the tube is filled from the test tube by pouring, then stoppered, and finally inserted into its properly numbered receptacle in the disc. We centrifugalize 10 minutes at a speed of 2,000 to 3,000 revolutions a minute.

To obtain the sediment with least disturbance, first remove the stopper at the inner or cream end, then, holding the tube with the cream end downward, remove the cream with a platinum loop and pour the milk out; lastly, still holding the cream end down, carefully remove the other stopper with the adhering sediment and smear the sediment evenly, with a drop of sterile water, over the measured space on the glass slide, rubbing the stopper directly on the glass until all the sediment is removed. Dry with gentle heat and stain with methylene blue.

The examination of a properly prepared milk sediment under the microscope with a $\frac{1}{13}$ oil immersion lens gives a very good idea as to the number of bacteria present. We began this examination with the intention of looking for pus and streptococci alone. The variation in the number of bacteria in different samples was, however, so apparent under the microscope that it suggested this form of examination as a more convenient and quicker method than plating for determining the bacterial content of milk, or at least for eliminating samples comparatively free of bacteria.

We determined to test the question thoroughly by comparing the microscopical estimate with the actual count as obtained from the plates. The comparison was carried out very carefully with over 2,200 samples, each sample being subjected to the double test, i. e., plating and centrifugalizing; the microscopic estimate was made before the plate was counted and an error of less than 1 per cent was made in passing (as below 500,000 bacteria to 1 c.c.) milks which in the plates showed above this limit. Over a third of the total error occurred in the first 420 samples, before the method was fully developed.

This method would be useless in examining very clean milks for certification, as the lowest limit of accuracy would probably be around the 100,000 to 1 c.c. mark. Perhaps, by obtaining the sediment from a larger amount of milk, the test could be made more delicate.

If the microscopic estimate is made before the samples are plated,

it is hardly necessary to plate those samples which are manifestly within the law's requirements. Besides such cases, many milks are obviously above the legal limit; these are plated in order to confirm the microscopic examination, and to have definite figures for legal purposes. Besides those entirely below and above, there are still others about which the examiner is in doubt. These of course need to be plated. Finally, of those apparently above the law, there are occasional samples which on plating run below it. It is still a question in such cases whether the plate or the microscope is the more correct. This group, although a very small one, makes the plating of high count milk more necessary, since it would be inadvisable for legal purposes to go into court on the microscopic estimate alone.

It is easy to determine, through the microscopic examination, what dilution will be necessary in plating, in order to ascertain correctly the number of bacteria present.

When examining the sediment for pus, since pus cells and dead leucocytes are identical, it is impossible to differentiate them under the microscope by the appearance of the individual cells. While it is not uncommon to find milks with these cells nearly absent, most milks have at least three or four such cells to the $\frac{1}{12}$ oil immersion field.

Opinions as to the number of such cells required to indicate pus differ. Professor Bergey, of the University of Pennsylvania,¹ says: "There is still no agreement among bacteriologists as to the number of cells in a specimen that will justify the diagnosis of the presence of pus. The number of cells in a field of the $\frac{1}{12}$ immersion lens is taken arbitrarily at 10. This number of cells per field may not always indicate pus, but it is believed that in the majority of instances it does indicate the presence of pus in milk derived from individual cows." Stewart, of Philadelphia, with the sediment from 1 c.c. of milk spread over a surface of 1 sq. cm., allows 23 cells to the $\frac{1}{12}$ immersion field before reporting pus. On account of the large amount of this milk with high cellular content at present in the markets, and the not wholly unreasonable doubt as to whether such milk is injurious to the public health, it has seemed fairest to all concerned, while fully recognizing that some abnormal condition

¹ *Bulletin 125*, Comwlth. of Penn., Dept. Agric., 1904.

must be present in the animal yielding such milk, to adopt a standard for market milk, for the present, allowing not over 50 cells to the $\frac{1}{12}$ immersion field (spreading the sediment from 2 c.c. of milk over 4 sq. cm.). So little of the sediment is seen at once with the high power that it is well to confirm the diagnosis of pus by making a thorough examination of the whole surface with a low-power lens, to determine how uniform a smear has been made. By using an eye-piece micrometer, ruled in squares, the relation of the area of one square to that of the $\frac{1}{12}$ immersion lens being previously calculated, a count may be made with the low-power lens.

Streptococci are, in our experience, seldom found to any great extent by direct microscopical examination. Occasionally a sample will be found crowded with long chains. More often streptococci, if present, are in the form of diplococci or very short chains. In any examination where streptococci, diplococci, or cocci are found in the sediment, and the plate from the same sample contains colonies resembling streptococci colonies in excess of a count of 100,000 to 1 c.c., we transplant these colonies to broth to see if chains will develop. We first make and record an estimate of the number of such colonies present, then transfer from 10 to 50 of them to broth, and grow for 24 hours at 37° C. Streptococci in small numbers are present in most market milk.

We condemn a milk for streptococci when these three tests are all positive:

1. Microscopic examination of the sediment showing streptococci, diplococci, or cocci.
2. The plate from the same sample showing colonies resembling streptococci colonies, in excess of 100,000 to 1 c.c.
3. The broth culture from these colonies showing streptococci alone or in great excess of the other bacteria present.

In conclusion, a word on the correlation of temperature and count. At first thought, one would always expect to find a high bacterial count in samples showing a high temperature, and a low count in low temperature samples, and if all the milk in question were of the same age and had been kept continuously at the temperature found, this would generally be the case. But it is easy to see that a high temperature might accompany a low count on milk only a few hours

old which had not been properly cooled, while a low temperature and a high count are frequently found together in milk recently cooled which previously for 12 to 36 hours had been kept warm, as is often the case during transportation as practiced in some parts of the country. Thus, by taking temperature alone it is impossible to state whether or not a milk is good from the bacterial standpoint, and if milk is judged by the temperature standard alone, much that is good may be condemned, and very much that is poor will be kept on sale. Taking temperatures is important, and tells us how the milk is being cared for at time of sampling. By making bacterial counts, we find how the milk has been cared for up to the present, any lack of cleanliness or care being recorded by the corresponding increase in bacteria. By centrifugalizing and examining the sediment, we can estimate the bacteria present, and detect milk which on account of disease or dirt is unfit for use as food.

All of these methods of examination have been carefully worked out with checks and controls. Realizing that there is still great room for improvement in many ways, we recommend them as a system which has been reasonably successful in practice, for city laboratory work where quick reports are needed as well as the accurate examination of large numbers of samples. We do not offer them as developed to a final point of simplicity and efficiency; our hope is only that their presentation here may serve to provoke discussion and to secure ultimate agreement on uniform and efficient methods.